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Original Article

Susceptibility to Focal and Global Brain Ischemia of Alzheimer Mice Displaying Aβ Deposits: Effect of Immunoglobulin

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ABSTRACT: Cerebral ischemia is a risk factor for Alzheimer's disease (AD). Moreover, recent evidence indicates that it is a two-way street as the incidence rate of stroke is significantly higher in AD patients than those without the disease. Here we investigated the interaction of ischemic brain insults and AD in 9-month-old ApdE9 mice, which show full-blown accumulation of Aβ deposits and microgliosis in the brain. Permanent occlusion of the middle cerebral artery (pMCAo) resulted in 36% larger infarct in ApdE9 mice compared to their wild-type (wt) controls. This was not due to differences in endothelium-dependent vascular reactivity. Treatment with human intravenous immunoglobulin (IVIG) reduced the infarct volumes and abolished the increased vulnerability of ApdE9 mice to pMCAo induced brain ischemia. When the mice were exposed to global brain ischemia (GI), an insult of hippocampal cells, ApdE9 mice showed increased neuronal loss in CA2 and CA3 subregions compared to their wt controls. GI was associated with increased microgliosis, astrogliosis, infiltration of blood-derived monocytic cells, and neurogenesis without clear differences between the genotypes. IVIG treatment prevented the GI-induced neuron loss in hippocampal CA1 and CA3 regions in ApdE9 mice. IVIG treatment increased microgliosis in wt but not in ApdE9 mice. Finally, GI induced 60% reduction in the hippocampal Aβ burden in ApdE9 mice, which was not affected by IVIG treatment. The results indicate that the AD pathology with Aβ deposits and microgliosis increases ischemic vulnerability in various brain areas. Moreover, IVIG treatment may be beneficial especially in patients suffering from both acute ischemic insult and AD.

Key words: Alzheimer's disease, ApdE9 mice, global ischemia, hippocampus, IVIG treatment

Alzheimer's disease (AD) is the most common form of dementia among the elderly. The major pathological hallmark of AD, extracellular deposits of amyloid-β (Aβ) protein are formed from amyloid β-precursor protein (AβPP) by sequential splicing by β- and γ-secretases. This amyloidogenic pathway leads to formation of amyloid peptides of variable length, from which the 42 amino acid residue peptide is the main component of amyloid deposits [1]. Despite the acknowledged role of Aβ in the progression of AD, it is a more complex condition than a single disease with several factors contributing to its pathology [2-8]. Vascular risk factors, stroke and heart disease are associated with prevalence of AD and often co-exist in humans [2-8]. Moreover, recent clinical and experimental studies suggest that AD patients have increased risk of stroke [2-8], suggesting that the interaction of AD and ischemic brain insults might be bi-

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directional. The exact mechanism how these conditions contribute to the progression of AD is unknown.

Vascular dementia is the second most common form of dementia after AD [9]. It is caused by problems in the supply of blood to the brain because of a stroke or damage to small cerebral arteries. About 10 % of people with dementia have a type known as mixed dementia. In fact, clinicopathological studies suggest that some 30 % of patients with diagnostic criteria for AD have vascular pathology [10], while up to 90 % of AD patients were found to have cerebrovascular lesions upon autopsy [9].

Young adult transgenic mice over-expressing APP are more vulnerable to focal ischemic insults and have larger brain damage even without presence of Aβ deposits [11,12] or AD-associated microgliosis. APP695 Swedish mutation expressing mice have high levels of Aβ40, which directly increases oxidative stress in cerebral blood vessels and induces disturbances in endothelium dependent vascular reactivity [13-15]. This cerebrovascular dysfunction can be reproduced in wt mice by topical application of Aβ40, but not Aβ42 [13]. After focal ischemia, this impairment results in reduced collateral blood flow in areas adjacent to ischemic core and larger ischemic lesions [11]. However, mice over-expressing human wt APP751 show no vascular impairment, but increased vulnerability to focal ischemia. In addition, vulnerability of these mice is not related to age or cognitive deficits but increased microglial activation and inflammation. Moreover, an anti-inflammatory drug abolished increased vulnerability to focal ischemia [5,12].

Stroke and head trauma have been shown to increase accumulation of APP in the brain due to damage of the axonal transport [16,17], and it is suggested that brain insults enhance amyloidogenic processing of APP which could lead to buildup of Aβ deposits and eventually to AD [2-8]. However, some previous studies in transgenic animals have not been able to show changes in amyloid levels after ischemic insults. To investigate the effect of ischemic brain insults on the progression of AD pathology and overall interaction of AD and ischemic brain insults, we used transgenic ApdE9 mice at the age of 9 months when these mice have clearly elevated Aβ42 and Aβ40 levels and readily detectable amyloid deposits in hippocampus and cortex with associated microgliosis [18-20] and exposed them to focal and global brain ischemia (GI). We found that 9-month-old ApdE9 mice have normal cerebrovascular reactions but larger lesions after permanent middle cerebral artery occlusion (pMCAo). However, the treatment with a single intravenous immunoglobulin (IVIG) injection was able to abolish the ischemic vulnerability of ApdE9 mice. The ApdE9 mice also showed altered regional vulnerability of hippocampal pyramidal neurons to GI and marked decrease in hippocampal amyloid load when assessed five weeks after GI. The treatment with IVIG prevented the GI-induced infiltration of vascular inflammatory cells in ApdE9 but not in wt mice, did not affect GI-induced neurogenesis, but modified microgliosis in wt mice and astrogliosis in ApdE9 mice.

MATERIAL AND METHODS

Animals

The APP/PS1dE9 (ApdE9) mice were generated by co-injection of chimeric mouse/human APP695 harboring the Swedish mutation and human PS1-dE9 (deletion of exon 9) controlled by independent mouse prion protein promoter [21]. The ApdE9 mice were backcrossed to the C57Bl/6J strain for nine generations and male and female double transgenic mice and their wt littermates were used in this experiment. Twelve 9-month-old male mice were used for cerebrovascular reactivity measurement, forty 9-month-old female mice were used for focal cerebral ischemia and 92 male mice were used for GI. At this age, these mice have clearly elevated Aβ42 and Aβ40 levels and readily detectable amyloid deposits in hippocampal and cortical areas associated microgliosis [18-20]. For GI, enhanced green fluorescent protein (eGFP) mice [22] were used as donors for bone marrow transplantations to allow us trace infiltrating blood-derived monocytic cells in the brain. Animal experiments were conducted according to the Council of Europe legislation and regulations for animal protection and approved by the Animal Experiment Committee in State Provincial Office of Southern Finland.

Cerebrovascular reactivity

To measure vascular responses to acetylcholine, a small cranial window was drilled and dura was removed from 9 months old ApdE9 male mice and their wt littermates. Intact cerebral blood vessels were first superfused with Ringer suspension (pH 7.4, 37 ℃) to measure baseline and then with 10 μM acetylcholine in Ringer solution. Cerebral blood flow was monitored during the superfusions with laser Doppler (OxyFlo, Oxford Optronix, Oxford, UK) and relative changes in blood perfusion units was analyzed as in [11,12].

Bone marrow transplantation

At the age of approximately 6-months, 46 male ApdE9 double transgenic mice and their age and sex-matched littermate controls were anesthetized with a mixture of ketamine and zylazine and lethally irradiated heads protected with a single dose of 950 cGy (Varian 600C

Radiotherapy accelerator, 4 MV high-energy x-rays). Acrylic lucite beam spoiler was used to ensure sufficient surface dose while source to skin distance was set to 100 cm. The irradiation field were restricted to cover only body below cervical spine and confirmed based on change of the fur color to gray during the following days. On the next day the irradiated mice were transplanted with bone marrow cells (5 x 10^6 cells) isolated from 6 to 8 weeks-old eGFP donor mice [22] by tail vein injection as previously described by Malm et al., 2005 [23]. Briefly, the bone marrow (BM) was isolated from femur and tibia of the donor eGFP transgenic mice by flushing the bones with Hank's balanced salt solution (BioWhittaker, Cambrex Bio Science, Belgium) containing 10% fetal bovine serum (FBS) (Gibco, BRL/Life Sciences, Great Island, NY, USA). The cells were filtered through a 70-µm nylon mesh filter to obtain single-cell suspension. The cells were counted and suspended in HBSS containing 2% FBS for transplantation. Each recipient mouse received 5 x 10^6 cells in 0.3 ml by a tail vein injection.

Engraftment analysis by flow cytometry

The success of the transplantation was analyzed using flow cytometry by analyzing the ability of the transplanted eGFP-positive BM stem cells to produce new blood cells in the recipient mice four to six weeks after transplantation. Shortly, the blood samples were collected from the transplanted mice and stained with the following antibodies: R-Phycoerythrin (R-PE) conjugated Ly-6G and CD11b to detect granulocytes and monocytes, and Peridin chlorophyll-a protein (PerCP) conjugated CD45R/B220 and CD3e to detect B-cells and T-lymphocytes accordingly (all from BD Biosciences, NJ, USA). Data were evaluated with the Cellquest Pro™ software (BD immunocytometry system, CA, USA).

pMCAO and TTC-staining

Female ApdE9 mice and their wt littermates were anesthetized with 5% isoflurane (70% N_2O, 30% O_2) for induction and 2.0 % for maintenance. The rectal temperature was maintained at 37 ℃ with a heating pad during the surgery. The left MCA was exposed and electrocoagulated at the level of the inferior cerebral vein. 24h after the mice were sacrificed using CO_2 and decapitated carefully [12]. Vascular anatomy in the MCA territory was observed and notes of potential abnormalities were made. Brains were cut into 1 mm thick sections with brain slicer and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) staining. In this standard staining the infarcted area remains white as an indicator of collapsed energy metabolism and failure of the cells to convert TTC to a red colored formazan. The sections were then fixed with 3.65% formalin and pictures scanned using a flatbed scanner. The images were quantified using Image Pro Plus software.

Global ischemia

Chimeric male ApdE9 mice and their wt littermates were anesthetized with halothane (for induction of anesthesia 3%, for maintenance 1-2%; carrier gas 30% O_2/70% N_2O). Rectal temperature was maintained at 37 ℃ using a thermostatically controlled rectal probe connected to a homeothermic blanket system during the surgery. A small incision was made in the neck and both common carotid arteries were exposed and bilaterally compressed for 17 min [24]. After 17 min of ischemia blood flow was restored by removing the bilateral compression and the mice were left to recover in a heated 32 ℃ chamber for 24 h [24]. Sham mice underwen all the same procedures, except for arterial occlusion. The ApdE9 mice were subjected to GI approximately 2 months after the transplantation.

Treatments

As the first experiment, in the study of focal ischemia the mice received one intravenous injection of 1 g/kg of IVIG (Gammagard Liquid, Baxter, Germany) or saline 1 hour before the ischemia. As the second experiment, in the GI study, mice were treated 1 hour after the global ischemia with a single intravenous injection of 1 g/kg of IVIG or diluent, which we thought to serve as an even better control solution than saline.

Histochemistry

Five weeks after GI the mice were terminally anesthetized by pentobarbital sodium and transcardially perfused with heparinized saline. The brains were immersion-fixed in paraformaldehyde for 24 h and cryoprotected in 30% sucrose for 48 hours, followed by freezing in liquid nitrogen. The brains were cut in 20-µm-thick cryosections with 100 µm intervals. eGFP positive cells were calculated from the cryosections. Neuronal survival was assessed by NeuN staining (1:200; Millipore, MA, USA) from dorsal CA1, CA2 and CA3 areas. Activation and proliferation of astrocytes and microglia were assessed after immunostaining with GFAP (1:500; Dako, Glostrup, Denmark) and Iba 1 (1:200; Wako Chemicals GmbH, Neuss, Germany), respectively from hippocampal area. Human Aβ was detected with pan-Aβ antibody (1:300; BioSource, Invitrogen, Carlsbad, CA, USA) and newly born neurons by doublecortin (DCX, 1:200; Millipore). Primary antibody binding was visualized either by using an Alexa Fluor 568-conjugated secondary antibody

(1:200; Invitrogen) or by biotinylated secondary antibodies (1:200; Vector Laboratories, Burlingame, CA, USA), avidin–biotin complex, and by using nickel-enhanced diaminobenzidine (Ni-DAB; Sigma–Aldrich, St Louis, MO, USA) or NovaRED (Vector Laboratories) as substrate.

Cell counting, quantification and image analysis

Six sections at 100 μm intervals throughout the hippocampi were evaluated per animal in each experiment, both in cell counting and quantification of immunoreactive areas. Since stereological methods were not applied, cell counting results represent a relative estimate of the cell numbers rather than absolute values. Counting of eGFP-positive cells was done from the whole hippocampal area (HC) and subventricular zones (SV) present in the same sections. For the quantification of immunoreactive areas, the sections were imaged with an Olympus AX70 microscope (Olympus, NY, USA) attached to a digital camera (Color View 12 or F-View, Soft Imaging System, Germany) running an Analysis Software (Soft Imaging System). The immunoreactive areas were quantified using ImagePro Plus Software (Media Cybernetics, Silver Spring, MD, USA). For NeuN immunostainings sub-regions of the hippocampal pyramidal cell layer were selected and the relative area covered by immunoreactivity was quantified in identical regions of interests in all sections examined. The results are presented as percentage of stained cell body area per total quantified area and expressed as mean ± standard error of mean (SEM). Pan-Aβ, Iba1 and GFAP immunoreactivities were quantified from the whole hippocampal area, and the data are expressed as percent area of hippocampi occupied by the immunoreactivity and represented as the mean ± SEM. DCX immunoreactivity was quantified in the hippocampal subgranular zone of the dentate gyrus (DG). The data are presented as percent area of DG occupied by the immunoreactivity and represented as mean ± SEM. The area settings were kept constant throughout the quantification procedures.

Statistical analysis

Histological data were analyzed by using SPSS 14.0 or 17.0 software and reported as mean ± SEM. Grubb's test for outliers were used to determine statistical outliers from the data set. Linear mixed model with individual random effect was used to assess the *neuronal loss* between the study groups. This statistical analysis was chosen to take into consideration the fact that hemispheres are partially dependent on each other's blood flow. Also, C57Bl/6J mice are known to have variability in the occurrence of the posterior communicating arteries (PCommA) [24]. During ischemia, the hemisphere that receives blood circulation through PCommA suffers blood loss and consequent neural loss in smaller magnitude than the hemisphere that does not contain PCommA. In linear mixed model the casual occurrence of PCommAs is included into the analysis. Bonferroni's pair-wise comparison was used as post-hoc test to compare the groups. T-test was used to compare the treatment effects between diluent and GG treated groups. The statistical analysis for the determination of the effect of global cerebral ischemia and/or GG treatment on the brain Aβ deposition was done using ANOVA followed by Bonferroni posthoc test. The threshold for statistical significance was set at $p < 0.05$.

RESULTS

IVIG abolishes the increased susceptibility of ApdE9 mice to permanent focal ischemia

At the age of 9 months female ApdE9 mice and their wt littermates were subjected to pMCAo and the ischemic lesions were visualized with TTC staining 24 hours later. In ApdE9 mice, the volume of ischemic lesions was 36% larger than in wt mice (Fig. 1.) ($p < 0.005$). On a contrary to [11] the difference seen in the vulnerability to stroke was not likely related to vascular impairment, since both genotypes responded similarly to topical acetylcholine exposure, cerebral blood flow was equally increased 13.8% in wt and 13.9% in ApdE9 mice (n = 6).

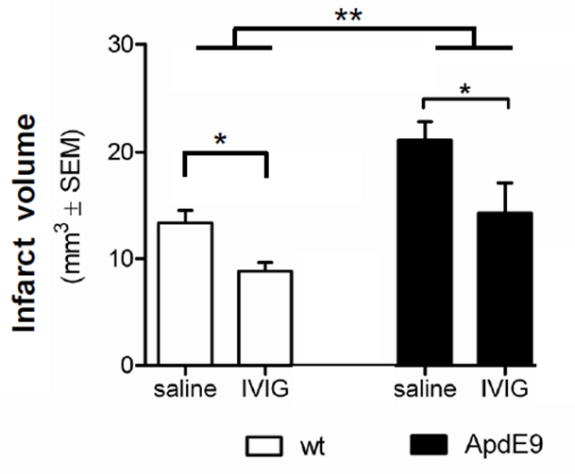

Figure 1. Nine-month-old ApdE9 mice have 36% larger lesions 24h after pMCA occlusion than their wt littermates ($p<0.05$) as analyzed with TTC-staining. Pretreatment with a single intravenous dose of IVIG reduced the infarct volume by 32% in aged ApdE9 mice to the level of non-treated wt mice, abolishing the increased vulnerability to acute ischemia. In addition, IVIG treatment reduced lesion size by 31% in wt mice. * denotes to $p < 0.05$ and ** denotes to $p < 0.01$, respectively.

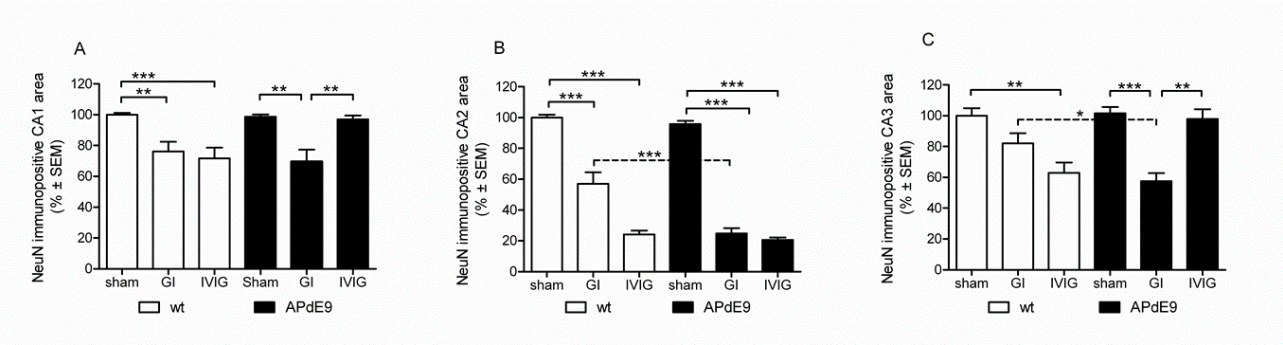

Figure 2. Neuronal survival measured with NeuN immunostaining from hippocampal areas 5 weeks after GI. 9-month-old ApdE9 mice have larger neuronal damage that their wt littermates in CA2 (**B**) and CA3 (**C**) areas, but not in CA1 area (**A**). GI causes significant neuronal damage in wt ($p<0.001$) and ApdE9 ($p<0.001$) mice in the CA1 area, but the difference between genotypes is not significant. However, the CA2 area is the most vulnerable to GI, since only 56% of NeuN-positive cells survive in wt mice ($p<0.001$). Moreover, in ApdE9 mice the damage is even more striking, only 24% of the CA2 cells survive ($p<0.001$), and therefore ApdE9 mice have 32% bigger neuronal damage than wt mice. The difference between genotypes is statistically significant ($p<0.001$). GI does not damage the CA3 area in wt mice ($p>0.05$) but causes significant neurodegeneration in ApdE9 mice ($p<0.001$). In wt mice, 82% of the CA3 cells survive 5 weeks after GI but in ApdE9 mice, only 57% of the NeuN positive cells survive. The neuronal vulnerability between genotypes is significant, since ApdE9 mice lose 25% more neurons from the CA3 area than wt mice ($p<0.05$) after GI. Mice were treated with 1 g/kg of IVIG or its diluent 1 hour after the global ischemia and neuronal survival was analyzed. In ApdE9 mice, the treatment protected CA1 and CA3 neurons from delayed neuronal damage as compared to diluent-treated mice. In the CA1 region, IVIG treated ApdE9 mice had 27.4% more neurons and in the CA3 region 40.3% more neurons than control mice. Linear mixed model, LSD as a post hoc test. In contrast, no protection was seen in the most severely damaged CA2 area. * denotes to $p < 0.05$, ** denotes to $p < 0.01$ and *** denotes to $p<0.001$, respectively.

IVIG contains naturally occurring auto-antibodies and has the ability to bind Aβ-peptides [25-27]. To study whether IVIG could abolish Aβ-mediated vulnerability in ApdE9 mice, ApdE9 mice and their wt littermates were treated with a single i.v. dose of 1g/kg IVIG or saline 1 hour before pMCA occlusion. As measured with TTC staining method at 24 h, IVIG had significant treatment effect in both genotypes by reducing the lesion volume by 32 % in ApdE9 mice and and 31% in wt mice (Fig 1). By IVIG treatment, the lesion volumes of ApdE9 mice were reduced to the level of saline treated wt mice, abolishing the increased vulnerability of ApdE9 mice to acute brain ischemia.

IVIG alters neuronal vulnerability to global ischemia in wt and ApdE9 mice and provides protection in CA1 and CA3 subfields of hippocampus in ApdE9 mice

The GI-induced neuronal damage was assessed 5 weeks after GI using NeuN staining and the survival of hippocampal pyramidal neurons was measured. GI was found to induce a significant damage to all hippocampal areas in wt mice. However, as in contrary to gerbil model of GI, the most vulnerable area in mice was the CA2 region, where only 56% of the cells survived (Fig. 2). The neuronal loss was marked also in the CA1 area, where 76% of surviving NeuN-positive cells were detected after GI, respectively (Fig. 2). Remarkably, the neuronal damage was more dramatic in ApdE9 mice. Although CA1 area was not significantly more degenerated in ApdE9 mice compared to their wt controls (69% vs. 76% cell survival), the degree of neuron loss in CA2 and CA3 areas upon GI was significantly higher in ApdE9 mice. In accordance with neuronal vulnerability in the wt mice, the most vulnerable region in the hippocampi of ApdE9 mice was also the CA2 area. Neuronal damage was extensive in this area as only 24% of the cells survived, whereas 57% of the CA3 cells survived from GI. In conclusion, ApdE9 mice lost 32% and 25% more cells in the CA2 and CA3 regions compared to their wt controls, showing that ApdE9 mice are more vulnerable to GI caused hippocampal neurodegeneration (Fig. 2).

To study whether IVIG treatment rescues hippocampal neurons from the enhanced vulnerability to GI, wt and ApdE9 mice were treated with a single intravenous dose of 1g/kg IVIG or its diluent 1 hour after the GI. As analyzed at 5 weeks after the GI, the treatment had reduced the GI-induced neuronal damage in ApdE9 mice, but not in wt mice (Fig. 2). The most vulnerable CA2 region was not salvageable by IVIG treatment, possibly due to high neuronal damage, whereas in the CA1 and CA3 areas a single dose of IVIG increased neuronal survival. Neuronal survival was significantly increased by 27.4% in the CA1 and 40.3% in the CA3 in IVIG treated ApdE9 mice compared to their diluent treated controls (Fig. 2).

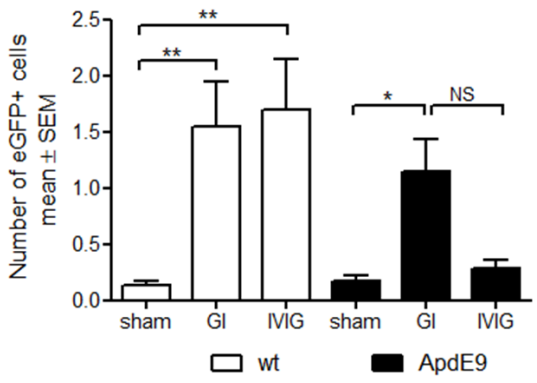

Figure 3. The number of infiltrated eGFP+ cells in hippocampal areas five weeks after GI. The hippocampi of sham operated mice was practically devoid of any eGFP positive cells, however, GI significantly increased infiltration of peripheral eGFP+ cells in wt and ApdE9 mice although the number of eGFP cells remained very low. * denotes to $p<0.05$ and ** $p<0.01$, respectively.

GI increases the infiltration of BM derived cells although the cell numbers remains low

The efficiency of eGFP+ cells to engraft the BM and to produce blood cells was analyzed by flow cytometry four to six weeks after the transplantation. The engraftment efficacy was equally good in both genotypes, 66% of the CD11b-labeled monocytes were eGFP positive, which is close to full body irradiation; only the levels of eGFP positive T and B cells were markedly lower [23] (Table 1).

Infiltration of peripheral cells after GI was measured by counting the number of eGFP positive cells from brain slices from hippocampal and subventricular areas. As a result, only occasional cells were detected in sham mice but GI significantly increased the number of eGFP+ cells in hippocampal area in both genotypes, although the number of infiltrating cells remained very low. The amount of infiltrating cells in the subventricular area was lower and the GI induced increase significant only in wt mice. Importantly, IVIG treatment did not have any effect on the number of eGFP+ cell in wt mice, but had a tencendy of reducing the number of infiltrated eGFP+ cells in ApdE9 mice after global ischemia (Fig. 3).

Neurogenesis is induced by global ischemia similarly in wt and ApdE9 mice and is not altered by IVIG treatment

GI is known to induce neurogenesis in the DG as a result of damage to hippocampal CA neurons [28]. To study the GI-induced neurogenesis in ApdE9 mice and their wt littermates, brain sections were stained with DCX. As a result, neurogenesis was equally induced in the subgranular zone of the DG in both wt and ApdE9 mice (Fig. 4). The treatment with IVIG had no additional effect on neurogenesis.

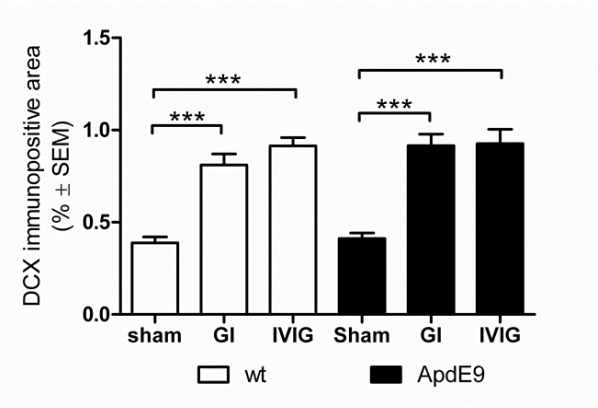

Figure 4. GI-induced neurogenesis in the subgranular zone of dentate gyrus measured as doublecortin immnoreactivity. GI enhanced neurogenesis in both genotypes. IVIG had no effect on neurogenesis. *** denotes to $p<0.001$, respectively.

Table 1. Engraftment analysis: percentage of eGFP+ blood cells 4 to 6 weeks after bone marrow transplantation

Genotype	B-Cells	Granulocytes	T-Cells	Monocytes
ApdE9	47.1 ± 1.9	67,5 ± 1.9	22.4 ± 1.3	66.5 ± 2.6
C57Bl/6J Crl	48.9 ± 2.2	70.6 ± 2.0	24.0 ± 1.3	67.0 ± 2.6
Total	48.0 ± 1.4	69.1 ± 1.4	23.2 ± 0.9	66.8 ± 1.9

Values are presented as mean ±SEM

Global ischemia reduced hippocampal Aβ burden

Next, the effect of GI on the AD-pathology progression was studied in ApdE9 mice. Five weeks after the GI the hippocampal area covered by pan-Aβ immunoreactivity was decreased by 60% ($p < 0.001$) (Fig. 5). The treatment with IVIG diminished the reduction to 43% but the effect of IVIG was not statistically significant compared to vehicle treated ApdE9 mice.

Activation of microglia and astrocytes

Microgliosis as analyzed by Iba-1 immunoreactivity in the hippocampi of ApdE9 mice was clearly visible as increased immunoreactivity surrounding the putative Aβ plaques (Fig. 6). As expected, GI triggered further increase in microgliosis in both genotypes (Fig. 6A). Interestingly, IVIG treatment significantly enhanced the GI-induced microgliosis in wt mice but not in ApdE9 mice. Opposite to microglia, no significant difference astrocytosis was observed in sham-operated ApdE9 mice compared to wt mice based on GFAP immunoreactivity (Fig. 6B). Astrogliosis was slightly but significantly increased by GI in ApdE9 mice but not in wt mice. In IVIG-treated GI mice the GFAP immunoreactivity was statistically not different from sham-operated wt and APdE9 mice, respectively. The figure panels C - H represent typical examples of Iba-1 immunoreactivity in the wt sham (C), wt GI vehicle (D) and wt GI IVIG (E) treated mice, ApdE9 sham (F), ApdE9 GI vehicle (G) and ApdE9 GI IVIG (H) treated mice. The figure panels I – N represent typical example images of GFAP immunoreactivity in the wt sham (I), wt GI vehicle (J) and wt GI IVIG (K) treated mice, ApdE9 sham (L), ApdE9 GI (M) vehicle and ApdE9 GI IVIG (N) treated mice.

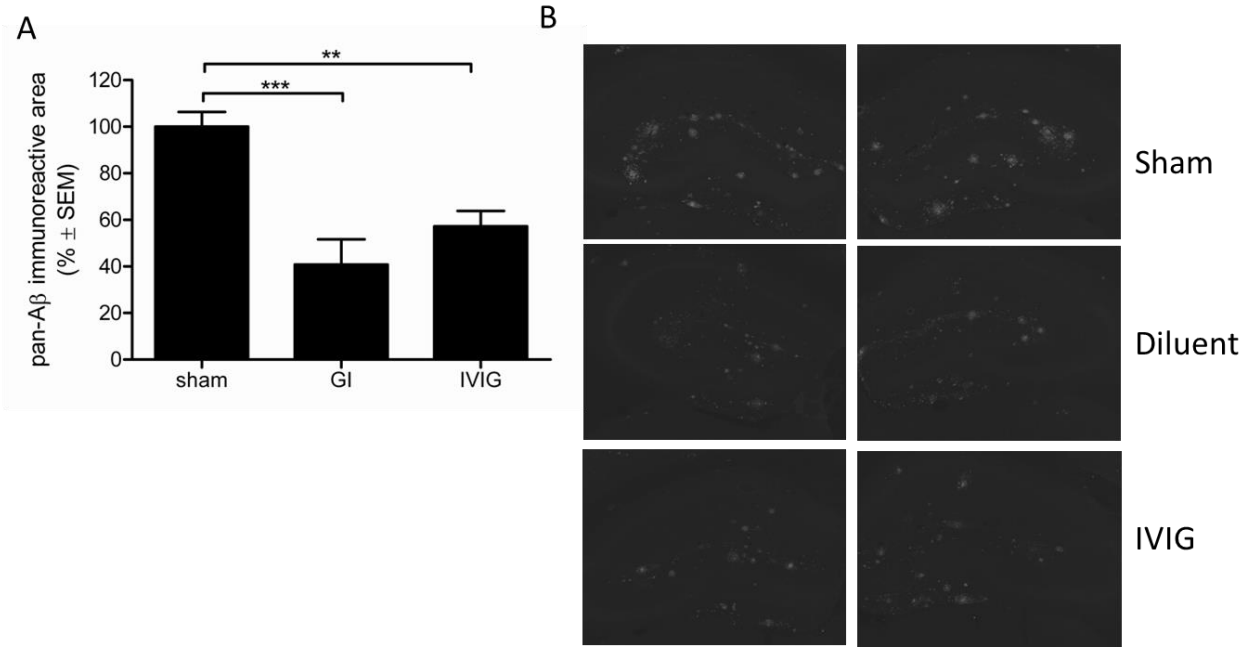

Figure 5. The effect of GI on amyloid load in ApdE9 mice as measured by Aβ immunoreactivity in the hippocampal area. ApdE9 mice were subjected to sham surgery or GI and treated with vehicle or IVIG. GI reduced the amount of hippocampal Aβ burden by 60% ($p<0.001$). IVIG treatment had no effect on the reduction of hippocampal Aβ. ** denotes to $p < 0.01$ and *** denotes to $p<0.001$, respectively.

DISCUSSION

Previous studies have shown that transgenic mice over-expressing wt or mutant APP or PS1 have increased susceptibility to ischemic brain insults at young age and in models where there is no accumulation of Aβ deposits or microgliosis in the brain [11,12,29]. This study shows for the first time that ApdE9 mice at the age of 9 months displaying frank Aβ pathology and microgliosis in the hippocampus and cortex, have increased vulnerability to both focal and global ischemic insults leading to increased damage to cortical and hippocampal structures,

respectively. This increased ischemic vulnerability appears not to be caused by dysfunctional endothelial cells, altered response in neurogenesis or infiltrating blood-derived monocytes. Pre-existing microgliosis in ApdE9 mice may have an impact to the increased ischemic vulnerability as microglia-derived inflammation can certainly impair neuronal survival after ischemic insults [12,15,30]. However, considering that previous studies have shown that AD transgenic mice over-expressing wt or mutant human APP or PS1 are more vulnerable to ischemic insults even at young age and without significant Aβ burden and microgliosis [11,12,29], it is likely that APP and PS1 mutations may result in compromised intracellular defense mechanisms in APP/PS1 mutant neurons. This is also the first study showing that IVIG diminishes the ischemic vulnerability of ApdE9 mice. The mechanism underlying the protection remains to be investigated but was not due to inhibition of glial cell activation, since we observed no changes in microglia or astrocyte activation upon IVIG treatment. Although the recent clinical trial testing IVIG in patients with AD did not support the benefit of IVIG on cognitive abilities in treated individuals (www.alz.org/aaic/releases_2013/tues_830am_ivig.asp), our results suggest that IVIG may be beneficial against ischemic insult in the context of AD-pathology. Indeed, it is of importance to note that a single intravenous injection of low-dose IVIG was sufficient to protect the cortex and hippocampus of ApdE9 mice after focal and global brain ischemia, respectively.

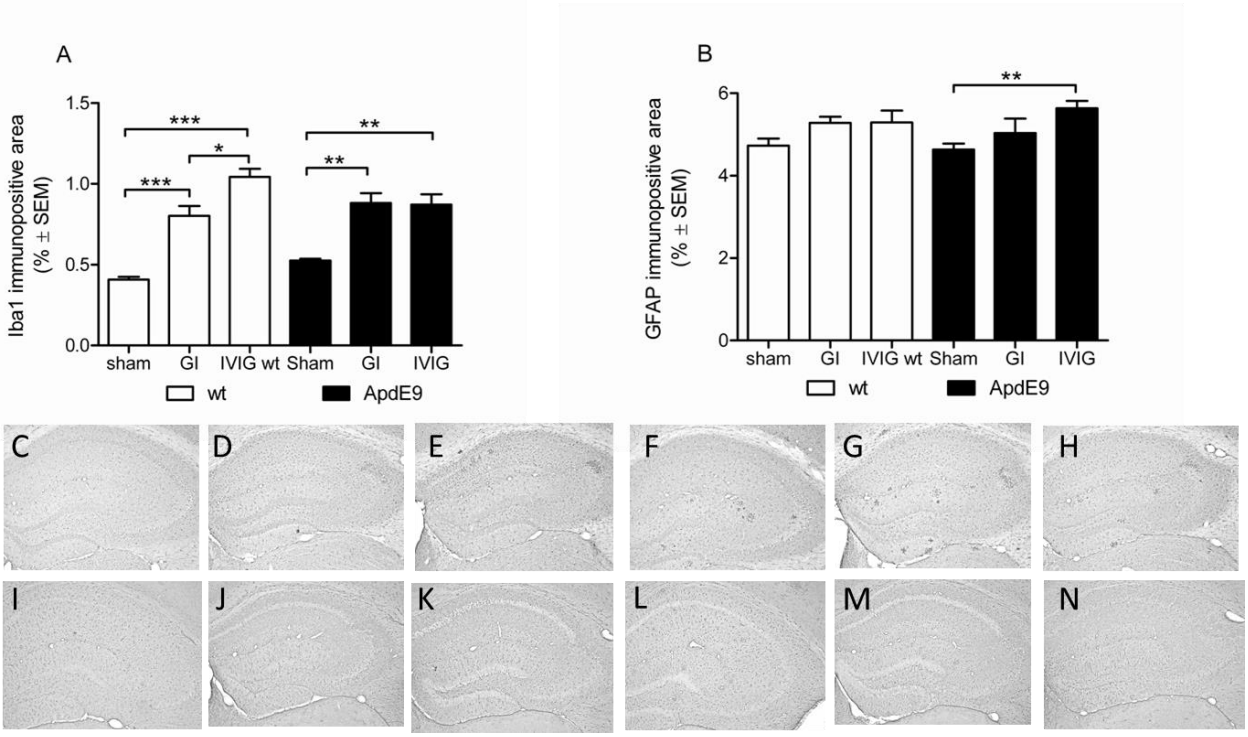

Figure 6. GI enhanced microglial activation (A) in both genotypes but not astrogial activation (B), measured as Iba1 and GFAP immunoreactivities in whole hippocampal area, respectively. IVIG had no effect on microgliosis (A), but increased slightly astrogliosis in ApdE9 mice (B). The figure panels C - H represent typical examples of Iba-1 immunoreactivity in the wt sham (C), wt GI vehicle (D) and wt GI IVIG (E) treated mice, ApdE9 sham (F), ApdE9 GI vehicle (G) and ApdE9 GI IVIG (H) treated mice. The figure panels I – N represent typical example images of GFAP immunoreactivity in the wt sham (I), wt GI vehicle (J) and wt GI IVIG (K) treated mice, ApdE9 sham (L), ApdE9 GI (M) vehicle and ApdE9 GI IVIG (N) treated mice. * denotes to $p < 0.05$, ** denotes to $p < 0.01$ and *** denotes to $p<0.001$, respectively.

AD rarely exists alone but is often associated with cerebrovascular defects [2-8,10,11]. Focal cerebral ischemia has been recognized to affect the severity of clinical symptoms of AD [3]. Indeed, ischemic insult has been shown to result in dramatic increase in the accumulation of both Aβ1-40 and Aβ1-42 peptides in human hippocampus and in rodent brain [31,32]. There is much less evidence of the pathomolecular processes in AD triggered by GI, which occur when the blood flow into the brain is temporarily ceased as e.g. in cardiac arrest. Here we found that GI-induced neuronal loss was significantly increased in CA2 (42 ±8 % and 77 ±3 % in wt and ApdE9 mice, respectively) and CA3 (19 ±6 % and 43 ±5 %) subregions of the hippocampus but not in CA1 (25 ±7 % and 31 ±8 %). Our previous studies employing wt C57Bl/6J mice to investigate hippocampal damage in GI have shown that the CA2 pyramidal cell layer of hippocampus suffers the most in wt mice [Kärkkäinen et al., in press, Koistinaho et al., unpublished]. As there is no difference in the Aβ burden or microgliosis between these hippacampal areas, the specifically increased vulnerability of CA2 and CA3 regions to GI induced neuron loss in ApdE9 mice appears to be unrelated to the extent of extracellular Aβ toxicity and glia-mediated inflammation. Thus, our data on GI-induced neuronal damage in the hippocampus is in line with the hypothesis that Aβ-related neurotoxicity, possibly by intraneuronal mechanisms, compromises the neuronal defense system against ischemia / hypoxia-reoxygenation-induced oxidative stress. We have previously shown that IVIG is directly neuroprotective in primary hippocampal neurons against Aβ induced toxicity [26] and IVIG has been shown to be neuroprotective in focal cerebral ischemia by reducing apoptotic cell death and inflammasome activation [33,34]. The Aβ pathology associated susceptibility to ischemic hippocampal damage was protected by IVIG in the CA1 and CA3 regions but not in CA2 region, possibly because the neuronal damage in CA2 might have been too extensive to be salvaged by administration of potentially neuroprotective IVIG after the insult.

Microglia are resident immune effector cells of the brain capable of phagocytosing harmful material including dead cells and protein aggregates such as Aβ deposits under certain circumstances [35,36]. The old dogma that microglial population is solely of embryonic origin has been challenged by BM chimera studies showing that microglial population, especially nearby capillaries, may be constantly replenished to a certain extent by blood-derived monocytes [23,37]. Importantly, blood brain barrier (BBB) damage has been shown to increase or even be required for CNS infiltration of BM-derived monocytic cells [37]. Kinetics of molecule transportation between the blood, CNS and CSF compartments is altered even in early AD alone [38-43], and the BBB is disrupted by ischemic events [30]. Moreover, the transgenic mouse models of AD differ in their BBB integrity [42-46]. In general, the mouse lines that deposit Aβ on the brain vasculature are more prone to have a leaky BBB [42-46]. The AD transgenic mouse line used in this study, the ApdE9 mice, do not deposit vascular Aβ to high degree compared to other models and the majority of Aβ is accumulated into the brain parenchyma as extracellular deposits. To study the GI and AD pathology –induced engraftment of blood-derived monocytic cells, the irradiation was restricted to the periphery of the body leaving the heads and thus BBB intact. The engraftment efficacy as judged by the percentage of eGFP-positive blood cells at 4-6 weeks after transplantation and 1 day prior to scarification was close to that achieved by full body irradiation in previous study [23] and only the percentage eGFP positive B- and T-cells was markedly smaller. The overall success of transplantation was similar both in ApdE9 and wt mice.

The Aβ burden in ApdE9 mice subjected to GI was remarkably decreased by 60% compared to sham operated mice at 32 days after GI. This corresponded to the GI induced increase in microgliosis. We have previously shown that BM-derived cells infiltrate into the brains of transgenic mice mimicking AD and seek to the vicinity of Aβ deposits [23]. This infiltration is massively enhanced by local LPS-induced inflammation, which also triggers the activation of endogenous microglia and results in concomitant reduction in hippocampal Aβ burden [23]. We thus wanted to examine whether BM-derived cells would influence the Aβ levels upon GI. Here we detected only occasional infiltrated cells in the brains of all the treatment groups. In our earlier studies the BM reconstitution was done using whole body irradiation and the observation time between the transplantation and the sacrifice was substantially longer. Whole body irradiation enhances the infiltration of cells into the brain parenchyma [47] and the restriction of the brain outside the irradiation in the current study is likely to account for the low number of detected eGFP positive cells. Although ApdE9 mice do not accumulate vascular Aβ, their BBB is somewhat compromised [48]. Overall, despite good BM reconstitution efficacy and the fact that GI induced significant increase in the infiltration of BM-derived cells in the brain, the overall number of eGFP-positive cells was very low in all mice indicating that 1) the GI model used does not induce overt BBB leakage and subsequent heavy invasion of peripheral cells into the CNS and 2) BM-derived cells do not account for the observed GI induced reduction in the hippocampal Aβ burden. Indeed, GI induced microgliosis or astrocytosis may account for reduced Aβ load via phagocytosis or production of amyloid degrading enzymes. Alternatively, GI may alter

BBB permeability to allow efflux of Aβ from brain to blood.

While AD has been reported to reduce endogeneous neurogenesis in mouse models of AD [49-52], cerebral ischemia conversely triggers it in specific brain regions [28]. In our study GI-induced neurogenesis in wt and ApdE9 mice to the same extent, suggesting that the possible difference in basal neurogenesis between wt and AD mice and the slightly different degree of ischemic hippocampal damage are not dominating factors in determining the injury-induced neurogenesis in the mouse hippocampus. Moreover, even though IVIG treatment fully protected the CA1 and CA3 subregions of the hippocampus of ApdE9 mice from ischemic damage, no difference in the level of hippocampal neurogenesis was observed. This is in line with previous studies suggesting that the GI-induced neurogenesis in the DG is not necessarily attributable to the extent of pyramidal neuronal loss [28].

In summary, ApdE9 transgenic mice displaying Aβ deposition and microgliosis mice have normal cerebrovascular reactions but larger lesions after focal and global brain ischemia. GI results in very mild infiltration of blood-derived monocytic cells and in ApdE9 mice reduces the hippocampal Aβ burden. Treatment with a single dose of IVIG can abolish the ischemic vulnerability of the ApdE9 mice, suggesting significant therapeutic potential of IVIG in ischemic brain insults and vascular dementia.

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